High-Gradient Magnetic Affinity Separation of Trypsin from Porcine Pancreatin

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Received 5 September 2001; accepted 4 February 2002

DOI: 10.1002/bit.10285

Abstract: We introduce a robust and scale-flexible approach to macromolecule purification employing tailor-made magnetic adsorbents and high-gradient magnetic separation technology adapted from the mineral processing industries. Detailed procedures for the synthesis of large quantities of low-cost defined submicron-sized magnetic supports are presented. These support materials exhibit unique features, which facilitate their large-scale processing using high magnetic field gradients, namely sufficiently high magnetization, a relatively narrow particle size distribution and ideal superparamagnetism. Following systematic optimization with respect to activation chemistry, spacer length and ligand density, conditions for preparation of effective high capacity ($Q_{max} = 120$ mg g^{-1}) strongly interacting ($K_d < 0.3$ μm) trypsin-binding adsorbents based on immobilized benzamidine were established. In small-scale studies ≈95% of the endogenous trypsin present in a crude porcine pancreatin feedstock was recovered with a purification factor of ≈4.1 at the expense of only a 4% loss in α-amylase activity. Efficient recovery of trypsin from the same feedstock was demonstrated at a vastly increased scale using a high-gradient magnetic separation system to capture loaded benzamidine-linked adsorbents following batch adsorption. With the aid of a simple recycle loop over 80% of the initially adsorbed trypsin was recovered in-line with an overall purification factor of ≈3.5.
 Biotechnol Bioeng **79**: 301–313, 2002.

Keywords: affinity separation; batch adsorption; high-gradient magnetic separation; primary capture; trypsin

INTRODUCTION

Many biotechnology companies are looking for new ways to make their manufacturing processes more cost effective and for many processes the key to achieving this may lie in streamlining product recovery and purification steps which can account for a large percentage of manufacturing costs (Spalding, 1991).

One approach receiving considerable attention is to selectively capture soluble target products directly from unclarified bioprocess feedstocks thereby fusing three classical steps – clarification, concentration, and initial purification – into a single unit operation. The most promising and practical of these is currently expanded bed adsorption (EBA), a technology based on fluidization employing densified chromatographic supports of defined size and density (Barnfield Frej et al., 1995; Hannson et al., 1994; McCreath et al., 1995; Thömmes, 1997). Successful processing by EBA has so far been demonstrated with a wide variety of target molecules and biological process feedstocks and in the last few years has found application in a number of pharmaceutical companies (Kasumasa et al., 1996). However, in common with most packed-bed chromatography materials, which over the years have been optimized for use with highly clarified feedstocks, most EBA supports presently in use are porous.

A potential constraint of any porous adsorbent is its propensity to become 'plugged' with biological foulants and/or suspended solids. Once fouled, in addition to diminishing access of the target protein to a large fraction of potential binding sites the difficulties in removing such substances can be acute. In contrast, nonporous support particles are less prone to fouling and are easier to clean than their porous counterparts and may thus be better suited to the task of product recovery from dirty fouling feedstreams (Eveleigh, 1978; Halling and Dunnill, 1979a, 1979b, 1980; Munro et al., 1977). Coated nonporous adsorbents of submicron dimensions exhibit all the advantages of larger nonporous supports, while at the same time possessing surface areas and therefore binding capacities greater than those of typical macroporous adsorbents (Anspach et al., 1989; Groman and Wilchek, 1987; Morgan, 1996; O'Brien et al., 1996, 1997; Zulqarnain, 1999). However, using support materials of such small size necessitates that adsorption takes place in

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Contract grant sponsors: Danish Technical Research Council (Statens Teknisk-Videnskabelige Forskningråd, STVF)

stirred tanks. The efficiency of any batch binding process relies critically on two factors, namely ensuring: (1) efficient adsorption of the target molecule (or contaminants); and (2) rapid separation of the "product-loaded" adsorbent from the crude suspension feedstock.

Magnetic particle-based separations are fast, gentle, and compatible with complex biological suspensions such as whole blood, milk, and cell disruptates and have consequently become increasingly popular in laboratories, especially for routine handling of large numbers of samples in molecular biology, cell sorting, and diagnostic applications. However, most commercial applications of magnetic separation technology so far have been at truly large scales of operation. For example, magnetic separation is of great economic importance to the clay, minerals, and nuclear processing industries and has also found efficient application in municipal and waste water treatment (Setchell, 1985; Svoboda, 1987). Developments in magnetic separation technology over the last 40 years (Svoboda, 1987; Watson, 1973) have gone largely unnoticed by biotechnologists, and so, not surprisingly, few successful attempts to scale up magnetic operations in biotechnology have been reported (Moffat et al., 1994; Williams et al., 1992). The power and efficiency of modern low-cost high-gradient magnetic separation equipment is especially useful and is probably the only practical way of rapidly recovering adsorbent particles of submicron dimensions from large volumes of crude liquors containing suspended biological particulates of roughly similar size.

Here we introduce a robust and scaleable approach to macromolecule purification which relies on: (1) initial capture of a target product onto tiny customized magnetic adsorbent particles; followed by (2) rapid collection of the "loaded" supports from the suspending liquor using powerful magnetic field gradients. We first describe the manufacture and characterization of large quantities of suitable magnetic support particles. We then go on to demonstrate how effective affinity adsorbents can be prepared using conventional coupling chemistry employed for commercial chromatography media and optimized with respect to specific product capture. Finally, we describe the successful application of a pilot-scale high-gradient magnetic separation system for product recovery from an unclarified bioprocess liquor. In the work described herein, we illustrate the whole integrated magnetic separation approach using benzamidine as a classical affinity ligand for the recovery of trypsin from a crude porcine pancreatin feedstock.

MATERIALS AND METHODS

Materials

The expanded metal matrix, KnitMesh type 9029, employed in high-gradient magnetic separation experiments was received as a gift from Colin Barnes (KnitMesh, South Croydon, Surrey, UK). The salts, iron (II) chloride hexahydrate and sodium carbonate were supplied by Merck (Darmstadt, Germany), while iron (III) chloride tetrahydrate was purchased from Fluka (Buchs, Switzerland). Methanol (GPR grade) and glycerol were supplied by BDH Laboratory Supplies (Poole, Dorset, UK) and J.T. Baker (Deventer, The Netherlands), respectively. The following materials were purchased from the Sigma Chemical Company (St. Louis, MO): trypsin from bovine pancreas (EC 3.4.21.4, Sigma type I, T 8003); α-amylase from porcine pancreas (EC 3.2.1.1, Sigma type VI-B); porcine pancreatin (P 1500); 3-aminopropyltriethoxysilane; glutaraldehyde (Grade I); epichlorohydrin; 1,4-butanediol diglycidyl ether; sodium borohydride; *p*-aminobenzamidine; ethanolamine and *N*-benzoyl-L-arginine-4-nitroanilide hydrochloride. The AMYL amylase assay was from Boehringer Mannheim (Mannheim, Germany) and fungal amylase standards were provided by Novo–Nordisk A/S (Bagsværd, Denmark). Bicinchoninic acid (BCA) Assay Reagent was obtained from Pierce Ltd. (Rockford, IL) and 12% homogeneous Phast^{TM} gels were purchased from Amersham Pharmacia Biotech, Uppsala, Sweden). Materials not listed here were supplied by the Sigma Chemical Company (St. Louis, MO).

Magnetic Particle Separation

At all stages of adsorbent manufacture and during use, powerful neodymium-iron-boron permanent magnet blocks (Danfysik A/S, Jyllinge, Denmark) and side-pull racks (PerSeptive Biosystems, Framingham, MA) were employed for particle recovery. At larger scales of operation magnetic particle recovery was achieved by high-gradient magnetic separation and a schematic representation of the HGMS system used in this work is illustrated in Figure 1. A specially designed water-cooled 1.2 Tesla electromagnet (Danfysik A/S, Jyllinge, Denmark) powered by a Danfysik System 8000 magnet power supply 858 (100 A, 52 V) lies at the heart of the rig. The magnetic flux density, B, in the air-gap was fine-tuned to a required value with the aid of Lakeshore model 410 gaussmeter (Lakeshore, Westerville, OH) fitted with a transverse probe. A collection chamber constructed of perspex with cone-shaped ($\alpha = 83°$) end pieces was positioned vertically between the plane pole shoes (set 5 cm apart) of the electromagnet (i.e., perpendicular to the applied field). Flow entering and leaving the chamber was distributed via perspex plates drilled with 30 holes of 2.5 mm in diameter. The working volume (16 mL, 20 mm long × 32 mm diameter) of the filter was filled with woven wire mesh (KnitMesh type 9029) composed of 430 stainless steel fibres of ≈110 μm thickness occupying 7% of the total working volume of the filter chamber (i.e., giving a voidage of 0.93). A Masterflex L/S^{TM} Easy-Load peristaltic pump (Model

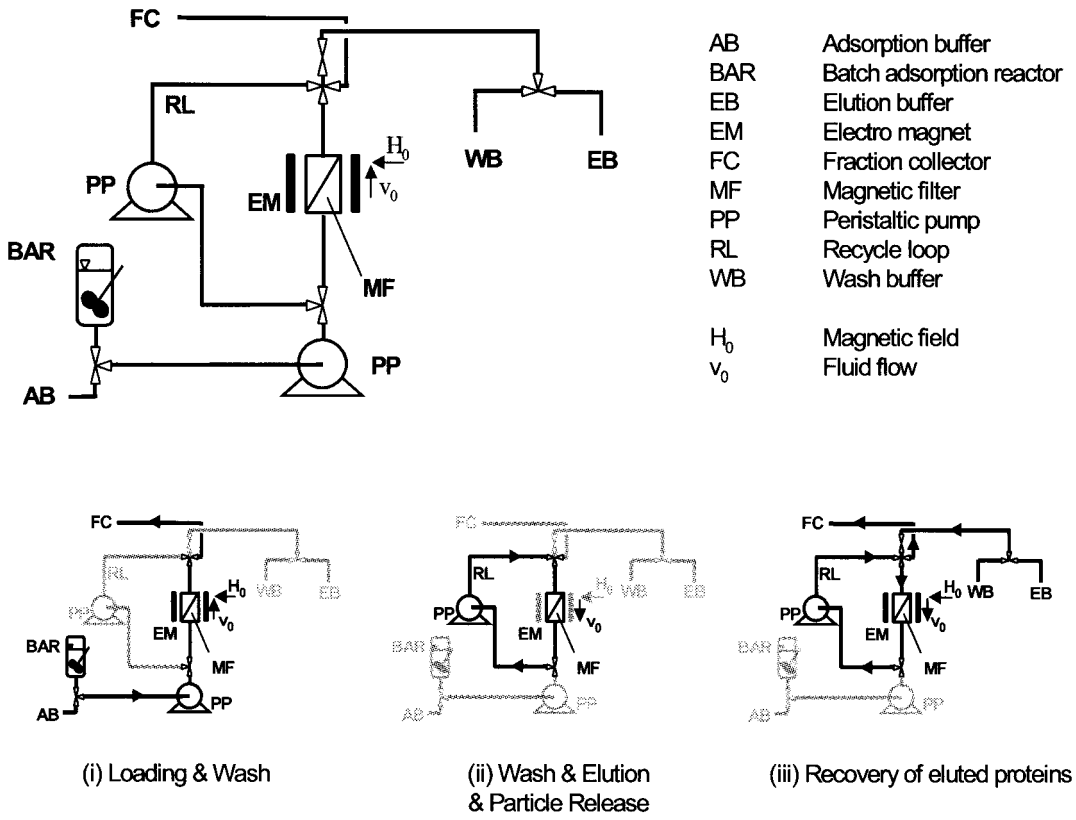

Figure 1. Basic system set-up for high-gradient magnetic separation.

7518-00, Cole Palmer Instrument Company, Vernon Hills, IL) was used for loading magnetic particles into the collection canister and for flushing them back out of the system at the end of experiments. Washing and elution operations were performed in the recycle loop (see Fig. 1) and employed a second Masterflex peristaltic pump. During experiments all fractions were collected with a SuperFrac fraction collector (Amersham Biosciences, Uppsala, Sweden) fitted with high flow adapters. The separation system's liquid flow path is controlled via an array of three-way solenoid switching valves (Bürkert-Contromatic A/S, Herlev, Denmark) driven off LabVIEW software (LabVIEW, National Instruments, Austin, TX).

Large-Scale Preparation of Coated Superparamagnetic Supports

Preparation of Iron Oxide Crystal Starting Materials

Routinely, a freshly made 0.75*M* "mixed iron" (1 Fe^{2+}/ 2 Fe^{3+}) chloride salts solution (two parts) was filtered through 0.45 μm cellulose nitrate membranes and was then rapidly mixed together (within ≈10 s) with an equal volume (two parts) of 5*M* NaOH by simultaneously pouring both solutions into a beaker containing one part of vigorously mixed distilled water. The mixing was provided by a stainless steel two-bladed impeller rotating at ≈200 rpm driven by an overhead motor (Heidolph Elektro KG, Type RZR, Schwabach, Germany). A dark precipitate of "green rust" intermediate formed instantly (Schwertmann and Fechter, 1994) and after ≈300 s of ageing and mixing black–brown iron oxide crystals were allowed to settle out of solution on a permanent magnetic plate. The clear liquid above the magnetic sediment was pumped off and the magnetic crystals were washed repeatedly with large volumes of distilled water by cycles of resuspension, magnetic separation, and decantation until the pH of the washings dropped below 8. The iron oxide crystal slurry was subsequently washed once with 0.2*M* NaCl and then twice with methanol before finally resuspending in 99% methanol/1% water to give a suspension with a crystal concentration of ≈40 g L^{-1}.

Preparation of Polyglutaraldehyde-Coated Superparamagnetic Particles

Two hundred and fifty milliliter portions of the above crystal suspension were transferred to 2-L plastic beakers and homogenized for ≈300 s at 2000 rpm using a Polytron® PT-MR 6000 dispersing device (Kinematica AG, Littau, Switzerland) fitted with a Polytron® PT-DA 6030-6060 aggregate. Thereafter, 10 mL of 3-aminopropyltriethoxysilane (3-APTES) was added followed

by 5 mL of glacial acetic acid and the homogenization speed increased to 13,000 rpm for ≈600 s before reducing it to 6000 rpm for 2 h. The slurries were then transferred to glass beakers containing an equal volume of glycerol and were stirred gently using an overhead mixer (Heidolph Elektro KG, Type RZR, Schwabach, Germany) for ≈300 s to obtain a homogeneous mixture. The contents of the beakers were subsequently heated on a halogen hot plate (CK 111, Schott-Geräte GmbH, Hofheim a. Ts., Germany) under a nitrogen atmosphere in a fume cupboard. In the first instance the temperature of the reaction cocktails was raised to 110°C over a 1–1.5 h period to evaporate the more volatile components. Once this temperature was reached the glycerol/particle slurries were cured for 10 h with gentle stirring under nitrogen, before finally raising it to 160°C over 0.5 h to drive off excess silane. The silanized magnetic particle/glycerol slurries were then removed from the heat and allowed to cool to room temperature under an atmosphere of nitrogen with gentle stirring. The amine-terminated magnetic particles were recovered from the glycerol-suspending medium by five consecutive cycles of magnetic separation, decanting, and resuspension with distilled water. The resulting amine-terminated particles were then coated with polyglutaraldehyde as described by O'Brien et al. (1996) and Zulqarnain (1999).

Support Activation

Protocols for the epoxy-activation of coated nonporous perfluorocarbons (Morgan et al., 1996) and various magnetic materials (Morgan, 1996; O'Brien et al., 1996; 1997; Zulqarnain, 1999) have been described recently. In this study polyglutaraldehyde-coated magnetic particles (at 16.7 g L^{-1} or 25 g L^{-1}) were vigorously shaken at room temperature for 6 h in reaction mixtures containing 35 m*M* $NaBH_4$, 0.5*M* sodium hydroxide and either 5% (v/v) epichlorohydrin or 50% (v/v) 1,4-butanediol diglycidyl ether. The epoxy-activated supports were washed copiously with distilled water and finally with 20 m*M* $NaHPO_4$ (cont. 1*M* NaCl) pH 6.8, before adjusting the particle concentrations to 25 g L^{-1}.

Coupling of *p*-Aminobenzamidine (*p*-AB) to Activated Supports

In a preliminary set of studies various coupling conditions were compared for the preparation of suitable benzamidine-linked supports. Two different epoxy-activated starting materials were employed (epichlorohydrin and 1,4-butanediol diglycidyl ether activated magnetic particles) at concentrations of 25 g L^{-1} and *p*-AB (at a concentration of 20% w/v) was coupled to these supports under alkaline (0.1*M* NaOH, 0.8*M* Na_2CO_3 or 1.0*M* Na_2CO_3) reducing (35 m*M* $NaBH_4$) conditions for 24 h with shaking in a heating chamber (Holten Laminar A/S, Allerod, Denmark) maintained at either 37 or 60°C. The specific reaction conditions for the various benzamidine-linked magnetic particle preparations (i.e., types Ia, Ib, IIa, IIb, and IIc) are detailed in Table I. After coupling, the supports were subsequently washed with water and then incubated in 1*M* ethanolamine for 24 h at 4°C to block excess oxirane groups remaining on the benzamidine-linked supports. The "blocked" adsorbents were finally washed with water to remove excess ethanolamine, resuspended to give a particle concentration of 20–25 g L^{-1} and stored at 4°C prior to use in batch-adsorption studies.

Type IIb and IIc benzamidine-linked supports were selected for further optimization work. The conditions for preparation of these adsorbents were the same as those described above and in Table I, except that the

Table I. Impact of activation and coupling conditions on the trypsin-binding properties of benzamidine-linked magnetic supports.

Epoxy-activating agent (spacer introduced)	Reactive oxirane[a] (μmol g^{-1}) introduced by capping with:		Support type:[b] alkali/temperature used in coupling	Langmuir parameters for binding of trypsin[c]		
	NH_4OH	EDA		Q_{max} (mg g^{-1})	K_d (μ*M*)	Initial slope[d] (L g^{-1})
Epichlorohydrin (3 atoms)	44.6 ± 0.5	59.6 ± 12.5	Type Ia: 0.8*M* Na_2CO_3/60°C	121.43 ± 8.55	1.15 ± 0.20	4.44
			Type Ib: 1.0*M* Na_2CO_3/60°C	131.79 ± 13.06	1.22 ± 0.26	4.53
1,4-Butanediol diglycidyl ether (12 atoms)	13.8 ± 1.8	16.7 ± 1.0	Type IIa: 0.1*M* NaOH/37°C	141.62 ± 7.80	4.24 ± 0.42	1.40
			Type IIb: 0.8*M* Na_2CO_3/60°C	125.29 ± 3.15	0.30 ± 0.03	17.55
			Type IIc: 1.0*M* Na_2CO_3/60°C	137.52 ± 5.76	0.79 ± 0.07	7.31

[a]Oxiranes available for coupling were estimated by an indirect approach involving coupling of small amine capping reagents (ammonia, ethylene diamine) and subsequent determination of the amine content introduced using the TNBS assay (values are given as the mean ± sample standard deviation, σ_{n-1}).

[b]All of the above benzamidine-linked supports were prepared by coupling of *p*-aminobenzamidine (20% w/v) under alkaline (0.1*M* NaOH, 0.8*M* Na_2CO_3 or 1.0*M* Na_2CO_3) reducing (35 m*M* $NaBH_4$) conditions to epoxy-activated magnetic particles (25 mg mL^{-1}) for 24 h at either 37°C or 60°C (see text for details).

[c]Data from adsorption isotherms were fitted to the simple Langmuir model [Eq. 1] using the χ^2 minimization procedure of Microcal Origin software version 4.1.

[d]Initial slope = Q_{max}/K_d.

p-AB concentration in the coupling reaction was varied between 0 and 20% (w/v).

Small-Scale Trypsin-Binding Studies

Small-scale binding experiments were carried out with trypsin from bovine pancreas and porcine pancreatin. The latter is a cocktail containing many enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. Bovine trypsin was resuspended with a 50 m*M* sodium phosphate, pH 7.7 binding buffer, while porcine pancreatin powder was reconstituted with a binding solution composed of 100 m*M* Tris-HCl pH 7.5 buffer supplemented with 20 m*M* $CaCl_2$. Prior to batch binding experiments the different benzamidine-linked magnetic particle preparations were resuspended and equilibrated in the appropriate buffers. Test solutions (500 μL or 1 mL) of varying concentrations were then added to various amounts (0.1 to 2 mg) of adsorbents in screw-capped vials (Sarstedt, Nümbrecht, Germany). The support/protein mixtures were routinely incubated at 20°C for 0.5 h on an IKA VXR-S17 vibrating shaker (IKA Labortechnik, Staufen, Germany). Following batch binding, the supports were separated from the bulk phases and were analyzed for residual trypsin activity and protein content.

Recovery of Trypsin from Crude Pancreatin by HGMS

A large quantity (equivalent to 0.4 g dried weight) of type IIb benzamidine-linked superparamagnetic affinity supports was thoroughly equilibrated in binding buffer (100 m*M* Tris-HCl pH 7.5 buffer containing 0.5*M* NaCl and 20 m*M* $CaCl_2$). Following separation with a permanent magnet the wet particle cake (0.4 g dried weight) was resuspended with 0.4 L of 2 g L^{-1}crude unfiltered porcine pancreatin and mixed for 0.5 h in a stirred 1-L reactor.

After incubation the "particle/feedstock" suspension was pumped upwards through the high-gradient magnetic filter canister positioned vertically in a 0.4 T field (measured in the air-gap) at a superficial liquid velocity within the filter chamber of 3 m h^{-1}. After separation of the protein-loaded superparamagnetic adsorbent particles from the crude pancreatin feedstock and washing with 0.2 L of binding buffer, the magnetic field was switched off and the particles were released from the particle collection canister into the recycle loop filled with binding buffer to wash out any physically entrained and/or loosely adsorbed materials. The suspension was circulated around the closed loop for ≈300 s before recapturing the magnetic adsorbent particles in the canister by switching on the magnetic field. The washings were then pumped out to the fraction collector and the recycle loop and filter chamber were refilled with washing buffer to begin a new washing cycle.

Two further cycles of washing were performed as described above. In subsequent cycles of trypsin elution from the magnetic supports the recycle loop was first primed with elution buffer (0.1*M* glycine buffer pH 2.6 containing 20 m*M* $CaCl_2$) prior to releasing the magnetic adsorbents into the recycle line (by simultaneously switching off the field and changing the valve positions). The suspension was then circulated for ≈300 s in the recycle loop, before recapturing the particles and collecting the eluted fractions. This elution procedure was repeated three times in total. Finally, the eluted particles were recovered from the filter chamber and re-equilibrated for further use.

Analytical Methods

The bulk magnetic properties of different particle preparations were investigated at room temperature in a MicroMag 2900 Alternating Gradient Magnetometer (PMC, Princeton, NJ) and analyses of particle size distributions were performed with an ALV–5000E particle measurement system (ALV, Langen, Germany).

Particle concentrations were determined by a dry weight method. Sterile 0.45 μm filters (Gelman Sciences, Ann Arbor, MI) were pre-dried in a microwave oven at a power setting of 15% (≈90 W) for ≈600 s and were then cooled to room temperature in a dessiccator jar before weighing accurately on an analytical balance. Samples containing magnetic particles were then applied to these filters and filtered under vacuum before drying to constant weight in the microwave (typically for ≈0.25 h at a power of 90 W). After cooling in a dessiccator the filters were reweighed and the amount of particles were computed from change in weight of the filter.

Reactive amino groups on supports were determined using an assay adapted from that described by Halling and Dunnill (1979b) employing 2,4,6-trinitrobenzenesulfonic acid (TNBS). TNBS reacts with accessible amines on support surfaces by nucleophilic substitution to form an immobilized trinitrophenyl derivative. A subsequent treatment with hot alkali liberates picric acid, which is yellow, into the bulk phase. Briefly, the assay was performed as follows. Small samples (1 mg) of supports were resuspended in 1 mL aliquots of a solution containing 0.1% (w/v) TNBS and 3% (w/v) $Na_2B_4O_7$ and the particle suspensions were then shaken at 70°C for ≈300 s in a heated shaking chamber. The TNBS-treated magnetic particles were subsequently recovered by magnetic separation, washed with distilled water and then incubated with 1.5 mL of 1*M* NaOH at 70°C for 10 min. After cooling and magnetic separation the optical densities of aspirated hydrolysates were measured at a wavelength of 410 nm and compared with standards prepared by direct hydrolysis of TNBS (0–0.1%) in 1*M* NaOH at 70°C.

The relative number of freely accessible oxirane groups introduced onto the surfaces of polyglutaralde-

hyde-coated magnetic supports by epichlorohydrin- and 1,4-butanediol diglycidyl ether activation was estimated by an indirect two-step method. In the first step, small amine capping reagents (ammonia or ethylene diamine, EDA) were coupled to the epoxy-activated materials at 60°C, and in the second, the amine content so-introduced was determined by the TNBS assay described above. The capping reactions were carried out essentially as described by Hermanson et al. (1992) employing supports at a concentration of 25 g L^{-1}. Amination with EDA was performed for 24 h using a reaction cocktail containing: 20% (w/v) EDA; 35 m*M* $NaBH_4$; and 0.5*M* Na_2CO_3. Capping reactions with ammonia were carried out for 6 h using a 1*M* solution of NH_4OH containing 35 m*M* $NaBH_4$. Prior to assaying the reactive amine content introduced by capping, the supports were washed thoroughly with distilled water.

Trypsin activity was determined at 37°C by the method described by Erlanger et al. (1961) using *N*-benzoyl-L-arginine-4-nitroanilide hydrochloride (L-BAPNA) as the substrate. The assay solution was made immediately before use by mixing 1 part of a 0.04*M* solution of L-BAPNA in DMSO with 7 parts of a 67 m*M* sodium phosphate buffer, pH 7.6. Aliquots (400 μL) of the freshly made assay solution were then added to 50 μL volumes of samples and hydrolysis of the substrate to *p*-nitroaniline was followed at a wavelength of 405 nm for 480 s in a spectrophotometric robot (Cobas Mira, Roche Diagnostics, Switzerland). We define 1 unit of trypsin activity as the enzymatic activity needed for the conversion of 1 μmol of substrate per minute at 37°C.

α-Amylase activity was measured at 37°C in the Cobas Mira spectrophotometric robot using the AMYL assay kit. The activity of the enzyme was followed at 405 nm for 720 s by monitoring the appearance of *p*-nitrophenol. The α-amylase activities were expressed in FAU (fungal amylase units, an arbitrary measure used at Novo-Nordisk A/S). One FAU is defined as the amount of enzyme, which hydrolyzes 5.26 g of starch per hour (Carlsen, 1994).

Soluble protein content was routinely determined by the bicinchoninic acid (BCA) assay adapted for use with the Cobas Mira spectrophotometic robot. Protein contents were expressed as BSA equivalents.

The protein compositions of fractions collected during high-gradient magnetic separation studies with pancreatin were analyzed by reducing SDS-PAGE (Laemmli, 1970) in 12% (w/v) polyacrylamide gels. Prior to electrophoresis the proteins from 1-mL samples were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 25% (w/v). Following 0.5 h incubation on ice the protein pellets were recovered by centrifugation in a refrigerated microfuge operated at 3000*g* for ≈300 s (Ole Dich A/S, Hvidovre, Denmark). After removing the supernatants, the protein precipitates were then resuspended with 1-mL volumes of 5 m*M* HCl in acetone and recentrifuged as above. The supernatants were carefully discarded and the pellets were washed with 1 mL of cold acetone before air-drying in a desiccator to produce a fine powder suitable for resuspension in reducing SDS-PAGE sample buffer. All electrophoresis, staining, and destaining operations were carried out in a PhastSystem™ (Amersham Pharmacia Biotech, Uppsala, Sweden) comprising separation, control and development units. Images of appropriately loaded Coomassie blue-stained gels were captured with a Gel Doc 2000 Gel Documentation system (Bio-Rad, Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Preparation and Characterization of Magnetic Materials

The procedures for the large-scale manufacture of the APTES-coated magnetic supports used in this work were systematically optimized by Zulqarnain (1999). The preparation can be divided into two distinct stages. In the first, iron oxide crystal starting materials are prepared by the classical chemical precipitation reaction involving aqueous iron salts and strong base (Cornell and Schwertmann, 1996; Kobayashi and Matsunaga, 1991; Misawa et al., 1974; Molday and Molday, 1984; Schwertmann and Cornell, 1991; Shinkai et al., 1991) and in the second stage the crystals are simultaneously silanized and aggregated, using techniques adapted from Weetall (1993) and Whitehead et al. (1985) to form submicron-sized clusters composed of hundreds of crystal monomers.

With the aid of wide variety of techniques (X-ray diffraction, magnetometry, Mössbauer spectroscopy, differential scanning calorimetry, thermogravimetric analysis, infra-red spectrocopy, atomic force microscopy, and nitrogen gas adsorption) it was found that the Fe^{2+}/Fe^{3+} ratio chosen for the preparation of magnetic iron oxide crystals by chemical precipitation with NaOH is a critical factor in determining the surface area and magnetic properties of the resulting materials (Bui et al., 1998; Pankhurst and Thomas, unpublished results). A ratio of 1 Fe^{2+}/2 Fe^{3+} yields the best balance of surface area, magnetization and memory; producing 7–11 nm-sized iron oxide crystals with a BET surface area of >110 m^2 g^{-1}. The crystals are composed of cation-deficient magnetite (magnetite/maghemite combination) and contain substantial amounts of impurities, especially lepidocrocite (γ-FeOOH) and possibly ferrihydrite, which may be important in lending surface roughness and therefore enhanced surface area to the finished support particle products.

Organofunctional silane coupling agents, which are very widely employed for providing water-resistant bonding of polymers to silica and glass, can also be used

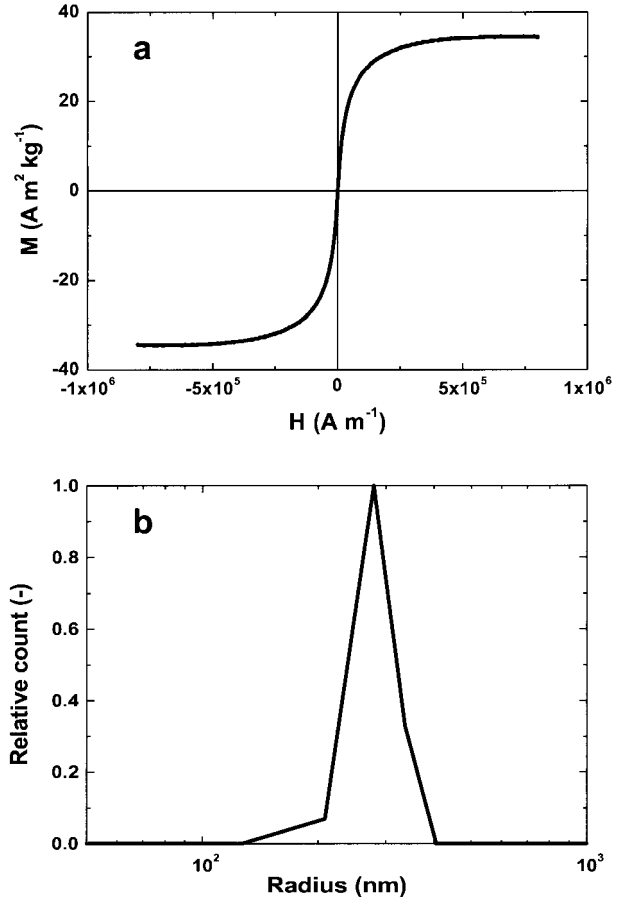

Figure 2. Magnetisation characteristics (a) and size distribution (b) of amine – terminated magnetic supports.

in a similar manner for the modification of metal and metal oxide surfaces (Plueddemann, 1991; Weetall, 1993). In common with glass, silicas and clay, the surfaces of common metals and metal oxides are hydroxylated. Under ambient conditions the population of hydroxyl groups on all of these materials equate to roughly one hydroxyl moiety per 0.5–1 nm^2 (Plueddemann, 1991).

A high degree of reproducibility is achieved during deposition of the silane coat, which is reflected by consistency in measurements of total nitrogen and reactive amine contents from batch-to-batch. The mean reactive (or accessible) amine content from five different amine-terminated preparations was determined as 97.4 ± 10.4 μmol g^{-1}, while estimations of the total APTES content from elemental nitrogen analysis were introduced roughly 18 times higher at 1.8 ± 0.3 mmol g^{-1} (n = 12). Taken collectively, these measurements suggest that most (≈95%) of the amines were buried and essentially inaccessible to TNBS. The cross-sectional area occupied by a single molecule of APTES has been estimated to be in the region 0.5–1 nm^2 (Naviroj et al., 1984; Schrader and Block, 1971). Assuming a specific surface area of 100 m^2 g^{-1} and an aminopropylsilane loading of 1.8 mmol g^{-1} we calculate an APTES density of 10 molecules per nm^2 which approximates to a uniform coverage equivalent to 10–20 close-packed monolayers and a film thickness in the region of only 5 to 20 nm (Plueddemann, 1991). From extensive studies on the stability of APTES-deposited films the optimum strength is observed for 10 to 20 equivalent monolayers. Increasing or decreasing the silane coverage significantly from this level results in a substantial drop off in film stability (Plueddemann, 1991).

Magnetic aggregation of particles is a well-studied occurrence (Parker et al., 1982; Svoboda, 1981; Tsouris et al., 1995; Wang et al., 1994) and in the mineral industry it is often used to enhance the recovery of ferromagnetic materials (Svoboda, 1987). However, when magnetic adsorbent particles are to be employed for protein recovery, permanent particle agglomeration should be avoided at all costs because it severely hinders adsorbent reusability and ease of product elution. Figure 2a shows a hysteresis loop from vibrating sample magnetometry (VSM) measurements of amine-terminated magnetic support particles prepared in this work. Important features to be derived from such a VSM profile are the characteristic saturation (M_s), remanence (M_r) and coercivity (H_c) values which decide the bulk magnetic properties and therefore the shape and openness of the loop (i.e., the degree of hysteresis). For the material in Figure 2a a maximum specific magnetization of 34.1 ± 0.3 A m^2 kg^{-1} was determined, but no residual magnetism could be detected and hence, the loop is completely closed. This is a classical example of superparamagnetic behavior (Dorman and Fiorani, 1992; Mørup and Tronc, 1994). Superparamagnetism, i.e., responsiveness to an applied magnetic field without any permanent magnetization, is an especially important property needed for magnetic particle-based separations because it enables repeated use of magnetic adsorbents and efficient product elution to be achieved. When a field is applied, superparamagnetic particles are magnetized and agglomerate through interparticle forces to allow facile separation. However, unlike ferromagnetic materials, when the field is subsequently removed the absence of magnetic memory inherent in superparamagnetic particles allows their easy redispersion to yield slow-settling suspensions possessing high-surface areas.

One of the most important parameters describing the efficiency of capture of magnetic particles by HGMS is the ratio of the magnetic velocity (v_m) to the applied fluid velocity (v_0) (Watson, 1973), and is defined by the following equation:

$$v_R = \frac{v_m}{v_0} = \frac{2\mu_0(\chi_s - \chi_f)M_w H_0 b^2}{9\eta a v_0} \qquad (1)$$

where μ_0 is the permeability of free space, χ_s and χ_f are magnetic susceptibilities of the support and fluid re-

spectively, M_w is the magnetization of the wire matrix, H_0 is the field strength of the applied magnetic field , η is the fluid viscosity and a is the wire radius and b is the radius of the particles. A value of ν_R much greater than unity is generally regarded as essential for strong capture of particles with a HGMS filter (Svoboda, 1987). Under the chosen operating conditions employed in this work values for ν_m and ν_0 of 0.27 m s^{-1} and 8.3×10^{-4} m s^{-1}, respectively lead to a ν_R of ≈330. The magnitude of this ν_R ratio illustrates the high suitability of the prepared superparamagnetic adsorbent particles for recovery by HGMS.

No noticeable changes in bulk magnetic properties or physical appearance were observed en route from the amine-terminated particles to the finished benzamidine-linked superparamagnetic adsorbents. Although we have not measured the depth of layers successively deposited on top of the amine-terminated supports by polyglutaraldehyde coating, epoxidation and ligand coupling reactions indirect evidence suggests that they must be ultrathin. Figure 2b shows a representative size-distribution profile for the amine-terminated particles used in this work. After successive polyglutaraldehyde coating, activation and ligand coupling, the mean particle size measured for four different batches of finished supports was very similar to that determined for two separate amine-terminated starting material (i.e., 0.68 μm ± 0.05 μm c.f. 0.86 μm ± 0.08 μm). Additionally, no significant changes in particle-settling rates or evidence of fast-sedimenting agglomerates were detected following further chemical elaboration performed on the amine-terminated particles. A relatively narrow particle-size distribution, as is exhibited by the adsorbents in this work, is desirable as it impacts beneficially on the predictability of magnetic particle collection by HGMS (Watson, 1994).

Optimization of the Preparation of Benzamidine-Linked Magnetic Supports

The results of a systematic study aimed at identifying optimal conditions for preparation of effective benzamidine-linked magnetic adsorbents for the recovery of trypsin are presented in Table I and Figures 3 and 4. In the first instance, two different epoxy activation methods were selected and the extents of activation introduced onto polyglutaraldehyde-coated particles were compared by an indirect method which involved capping of the reactive oxiranes with small amines (EDA and ammonia) and subsequent determination of the reactive amine content using the TNBS assay. Measurements with both capping agents revealed that epichlorohydrin introduced 3 to 4 times as many accessible epoxy groups as 1,4-butanediol diglycidyl ether activation. Capping with EDA yielded consistently higher amine contents than with ammonia. This is perhaps not surprising given the nearly sevenfold higher molar concentration of

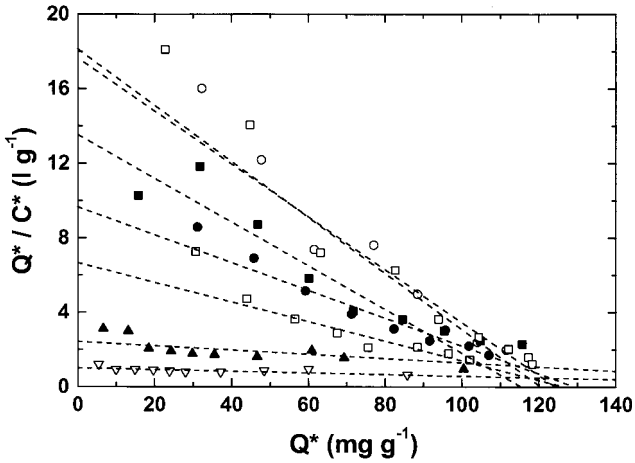

Figure 3. Scatchard plots for the binding of bovine trypsin to type 'IIb' benzamidine-linked superparamagnetic adsorbents. 1,4-butanediol diglycidyl ether activated supports were coupled with *p*-aminobenzamidine at various concentrations: (▽) 0% (w/v); (▲) 1% (w/v); (△) 5% (w/v); (●) 10% (w/v); (○) 15% (w/v); (■) 17.5% (w/v); (□) 20% (w/v). The data were first fitted to the Langmuir model (using the Microcal Origin 4.1 χ^2 minimization procedure) and the dashed lines in the above plots represent the resulting Langmuir fits to the data.

amine-capping groups employed. Various conditions for preparation of benzamidine-linked magnetic supports were then screened by studying the adsorption behavior of various adsorbent preparations using pure trypsin. The resulting data were fitted to the simple Langmuir (1918) model [Eq. (2)], in which Q^* and C^* represent the equilibrium concentrations of the adsorbed and bulk-phase target molecule, respectively, Q_{max} is the maxi-

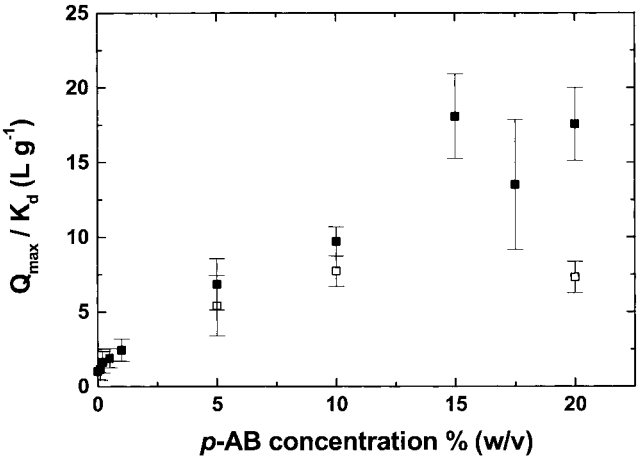

Figure 4. Effect of *p*–aminobenzamidine concentration in the coupling reaction on the initial slopes of isotherms for the binding of bovine trypsin to the resulting benzamidine-linked magnetic adsorbents. Type 'IIb' (■) and 'IIc' (□) supports. The initial slope (Q_{max}/K_d) values are derived from Langmuir fits to the adsorption data (using the Microcal Origin 4.1 χ^2 minimization procedure) and the boundary limits within which all experimental data points fall are indicated by the bars.

mum capacity for the adsorbed target and K_d is the dissociation constant.

$$Q^* = Q_{max} \frac{C^*}{K_d + C^*} \quad (2)$$

Although the mechanistic assumptions upon which the model are based are not met by the present system – as is the case for most systems dealing with protein adsorption – very good mathematical correlations between the experimentally obtained data points and Eq. (2) were obtained, which made quantitative comparisons of the different supports possible.

From the higher number of sites available for coupling small free primary amine-containing molecules on epichlorohydrin compared to 1,4-butanediol diglycidyl ether activated magnetic particles (see Table I), it follows that a higher benzamidine ligand density should be introduced onto the former. This said, it is clear that type IIb and IIc benzamidine-coupled affinity supports prepared from 1,4-butanediol diglycidyl ether activated materials exhibit much better trypsin binding behavior than those originating from the epichlorohydrin activated matrices (i.e., Ia and Ib). For example, relative to the type I benzamidine-linked supports the tightness of trypsin binding (which is reflected by the initial slope of the isotherm, i.e., Q_{max}/K_d) was >1.6 fold higher and nearly fourfold higher for types IIc and IIb benzamidine-coupled adsorbents respectively. The superior performance of the 1,4-butanediol diglycidyl-derived type IIb and IIc affinity adsorbents presumably reflects much improved ligand accessibility imparted by the 12-atom molecular spacer arm.

The very poor trypsin binding behavior observed for the IIa adsorbent is most likely attributed to low-level ligand substitution arising from inefficient coupling conditions (limited buffering capacity and reduced temperature). In subsequent optimization experiments only the type IIb and IIc epoxy activated magnetic materials were used. The *p*-AB concentration in the coupling reaction was systematically varied and resulting adsorbents were then employed in adsorption experiments with trypsin.

Figure 3 shows Scatchard (1949) plots derived from a series of binding experiments conducted with type IIb benzamidine-linked affinity supports, and in Figure 4 the initial slopes of adsorption isotherms are plotted against the *p*-AB concentration in the coupling reaction for both IIb and IIc affinity materials. Weak nonspecific adsorption of trypsin to supports was observed when low ($<1\%$) levels of *p*-AB were present in the coupling reaction mixture, but as the concentration was raised parallel increases in specificity (K_d) and effectiveness or tightness of binding (Q_{max}/K_d) of the resulting materials were observed until plateau values were reached at $>10\%$ *p*-AB for type IIc and $>15\%$ for type IIb affinity supports. Under these conditions very similar Q_{max} values of 120–130 mg g^{-1} were recorded for both types of matrices, but the dissociation constants were significantly different, i.e., 0.28–0.35 μM for type IIb and 0.70–0.79 μM for type IIc.

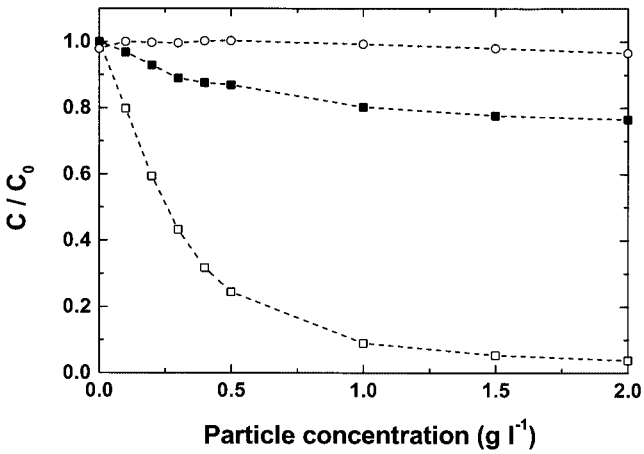

Figure 5. Selective removal of trypsin from crude porcine pancreatin using type 'IIb' benzamidine-linked magnetic adsorbents. Remaining trypsin activity (□), α-amylase activity (○) and protein content (■).

At all *p*-AB coupling concentrations employed the Langmuir binding parameters of the resulting type IIb affinity supports were markedly superior to those of the type IIc affinity materials (see Fig. 5). It appears that an elevated sodium carbonate concentration during coupling of *p*-AB has a detrimental impact on the trypsin binding performance of the resulting adsorbents. In studies with epichlorohydrin activated type I materials (see Table I) increase in the sodium carbonate concentration during coupling exerted no apparent effect on trypsin adsorption properties. The reasons for the differences observed above are currently unclear, but should be revealed when reliable methods for determination of immobilized benzamidine concentration are established. In all subsequent studies described herein we have employed type IIb benzamidine-linked magnetic materials which were prepared under fully optimized conditions.

Small-Scale Studies With Type IIb Benzamidine-Linked Magnetic Supports and Crude Porcine Pancreatin

To investigate the adsorption behavior of type IIb affinity support materials different concentrations of the adsorbents were incubated with crude porcine pancreatin and the levels of endogenous trypsin and α-amylase were subsequently monitored.

Figure 5 shows that capture of $>90\%$ of the trypsin present in the pancreatin feedstock could be achieved using a particle concentration of 1 g L^{-1} without loss in α-amylase activity from the liquid phase. A doubling in support concentration to 2 g L^{-1} raised the trypsin recovery to ≈95% at the expense of a 4% drop in bulk-phase level of α-amylase activity.

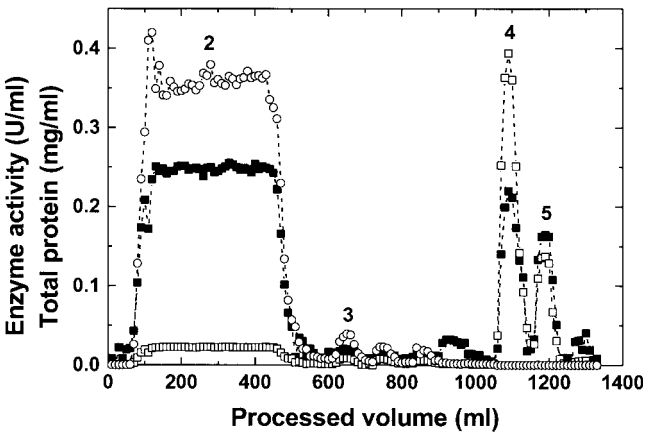

Figure 6. High-gradient magnetic affinity separation of trypsin from crude porcine pancreatin. Trypsin activity (□), α-amylase activity (○) and protein content (■). The numbers on the graph correspond to fractions analyzed in the SDS-polyacrylamide gel shown in Figure 8.

This loss in α-amylase is most probably attributed to entrainment in the magnetic particle cake rather than physical adsorption onto the adsorbent particle surfaces. The specific activities of the captured trypsin at the ≈90% and ≈95% recovery levels were respectively, 4.55- and 4.08-fold higher than that of untreated pancreatin.

High-Gradient Magnetic Affinity Separation of Trypsin from Crude Porcine Pancreatin

Figure 6 shows a profile of the preparative purification of trypsin using high-gradient magnetic affinity separation. Figure 7 shows the corresponding SDS-polyacrylamide gel analysis of selected fractions and Table II summarizes the data, which provide a qualitative evaluation of the performance of the purification process. In this study magnetic affinity supports and pancreatin were employed at concentrations of 1 g L^{-1} and 2 g L^{-1}, respectively (i.e., 0.5 g support of per g of pancreatin) to ensure a high level of recovery during batch adsorption (see Fig. 5). The batch-binding reaction was performed in a stirred reactor as opposed to the vibrating shaker used in small-scale studies and resulted in capture of only 76% of the endogenous trypsin present.

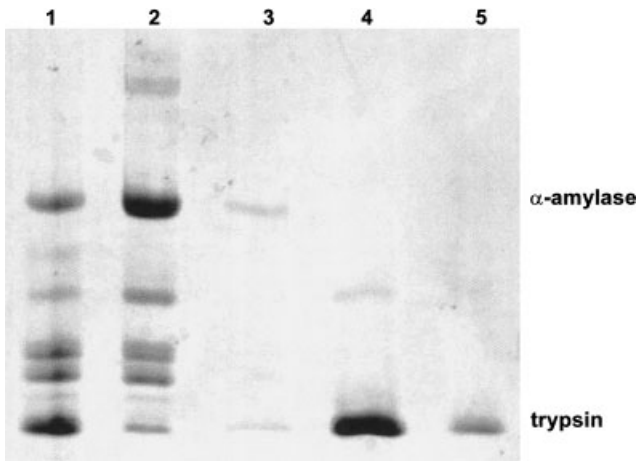

Figure 7. SDS-PAGE analysis of fractions collected at different stages of operation during high-gradient magnetic affinity separation of trypsin from crude porcine pancreatin (see Fig. 7). Lane 1, crude porcine pancreatin; Lane 2, during loading; Lane 3, during washing; Lane 4, 1st elution cycle; Lane 5, 2nd elution cycle.

Poor mixing on scale-up was the most probable reason for the reduced binding capacity observed. Nevertheless, the recovery was sufficiently high to allow proper evaluation of the potential of the process. In addition to the unbound trypsin fraction approximately 90% of the α-amylase and 78% of the total protein broke through during application of the support/feedstock mixture to the HGMS filter.

Following loading and wash-down, non-specifically adsorbed and physically entrained materials were removed from the filter with the aid of the recycle loop, which was filled with wash buffer. The canister and recycle loop were loaded with washing buffer and isolated to create a closed independent circuit. The externally applied field was then switched off and the magnetic particles were flushed out from the canister and around the closed circuit loop rapidly in a cyclic manner. This resulted in break down of magnetic support aggregates formed during adsorption to the steel filter matrix and release of physically entrained or loosely adsorbed nonmagnetic biological materials. The external field was then reapplied and the magnetic adsorbents recaptured in the magnetized filter matrix while the washing buffer and suspended nonmagnetic contents were collected and analyzed. Two further washing operations were performed using the recycle loop before preparing it for elution by chasing the wash buffer out the system with distilled water.

Material released during washing cycles could arise from either: (1) physical entrainment within the particle cake built up on the filter wires; (2) the release of nonspecifically adsorbed protein; and/or (3) desorption of specifically bound species due to the creation of a new binding equilibrium in the recycle loop. The composition of the pooled wash fraction (see Table II) was similar to that of the initial feedstock, albeit much diluted. This observation strongly suggests that the released protein primarily originates from nonspecific protein adsorption rather than physical entrapment or establishment of new equilibrium conditions within the recycle loop. Further, based on our measurements of particle density (≈5 g cm^{-3}) and used filter capacity in the above experiment of only 25 g particles per liter of matrix, together with an estimated voidage of the particle build-up of between 0.63 (Leitermann, 1985; Uchiyama et al., 1977) and 0.7 (Nesset et al., 1980; Nesset and Finch, 1980) we calculate a trapped interparticle volume in the whole filter canister of only 135–185 μL. This value is much too small to account for the

Table II. Summary of data from the recovery of trypsin from crude porcine pancreatin by high-gradient magnetic affinity separation (see Figs. 6 and 7).

Recovery step	Volume (L)	α-Amylase (Units)	Trypsin (Units)	Protein (mg)	% Yield	Purification factor
Feedstock	0.4	159.24	42.76	127.38	100	1
Loading and wash down	0.4 & 0.2	142.63	9.07	99.59		
Washing	0.3	4.54	1.29	3.44		
Buffer exchange	0.14	0.21	0.15	2.43		
Elution	0.3	0.08	26.39	22.73	62	3.46
Mass balance (%)		92.6	86.3	100.6		

measured enzyme and protein contents in the washing fraction. The subsequent chasing cycle with distilled water dislodged a small peak of protein (see Fig. 6 and Table II), but trypsin and α-amylase activities were barely detectable.

Specifically bound material was eluted from the magnetic adsorbents with 100 m*M* glycine-HCl buffer pH 2.6 containing 20 m*M* $CaCl_2$ in three successive cycles (see Figs. 6 and 7) using the recycle loop as described above. Over 80% of the initially adsorbed trypsin was recovered with a purification factor of ≈3.5 (see Table II). The volume of the recycle loop and canister in the present system predetermines the degree of product concentration that can be achieved as does operating the canister at close to its maximum particle holding capacity. In the present study only a quarter of the particle holding capacity of the filter was used and no attempts to optimize product concentration by reducing the volume of the recycle loop were made. Consequently, a low concentration factor of only 1.35 was recorded. This said, it is quite clear that significant product concentration should be achievable with HGMS systems operating under optimized conditions.

The recovery of captured particles from an HGMS filter is probably the most challenging problem encountered in pilot and production scale high-gradient magnetic separators (Svoboda, 1987). In this study nearly 80% of the support was recovered in-line using the recycle loop and high flow rates for flushing. Complete recovery of the adsorbents required dismantling the filter chamber for off-line cleaning. An increasingly important requirement in design of bioprocess equipment today is that efficient cleaning and sterilization procedures can be applied without dismantling the system. Accordingly, we are currently advancing the design of high-gradient magnetic separators to enable complete particle recovery to be carried out in-line and efficient cleaning – and sterilization – in-place (CIP and SIP) procedures to be implemented.

CONCLUSIONS

A common misconception held in biotechnology is that magnetic separations are not scaleable (see for example comments to this effect from Wils et al., 1997; and Schluep and Cooney, 1998). This is perhaps not surprising given that magnetic adsorbents available from biotechnology manufacturers are costly and can only be purchased in very small quantities. Parallel developments over the last 30 years in both bulk chemical synthesis of well-defined iron oxide materials and large scale magnetic separation technology have been largely ignored by biotechnologists and biochemical engineers and consequently few serious attempts to scale up magnetic separations in biotechnology have been made until now.

In the present study we have carefully considered advances in the above areas in the design of an integrated purification approach employing low-cost high-capacity superparamagnetic batch adsorbents and high-gradient magnetic separation technology. The consistent manufacture of stably coated supports possessing high-surface area, defined magnetic properties, and narrow particle size distribution in an easily scaleable and cost-effective manner is an essential prerequisite for the continuing development of large-scale HGMS-based purification processes. The base supports prepared and employed in this study fulfil many of these requirements.

The ability to use simple optimization procedures and conventional activation and ligand coupling methods already employed in the manufacture of commercial chromatographic adsorbents is clearly advantageous. With the aid of small-scale magnetic racks screening of conditions during support development and subsequent applications in biological feedstocks becomes both fast and easy. Moreover, once an optimized process is established in such small-scale adsorption experiments the data can be usefully employed to predict the overall process performance at large scales of operation using HGMS (Hubbuch, 2001; Hubbuch et al., 2001).

In conclusion, this pilot study has demonstrated the considerable potential of magnetic particle-based adsorption approaches for the direct recovery of specific macromolecular products from "dirty" biological process liquors. Future work will concentrate on extending the application of HGMS-based purification to a wide range of target products and feedstocks of varying complexity and concomitantly to advancing the design of HGMS equipment and adsorbents for bioprocessing tasks.

We are grateful to Søren Nielsen (BioCentrum-DTU, Technical University of Denmark) for carrying out the total elemental nitrogen analyses and Dr. Peter G. Weidler (Institute for Technical Chemistry, Forschungszentrum Karlsruhe GmbH, Germany) for helpful comments on the manuscript.

NOMENCLATURE

APTES	γ-aminopropyltriethoxysilane
BCA	bicinchoninic acid
BSA	bovine serum albumin
CIP	cleaning-in-place
HGMS	high-gradient magnetic separation
L-BAPNA -*N* -benzoyl	L-arginine-4-nitroanilide hydrochloride
p-AB	para-aminobenzamidine
SIP	sterilization-in-place
TCA	trichloroacetic acid
T	Tesla
TNBS	2,4,6-trinitrobenzenesulfonic acid
U	unit of enzyme activity

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